The Synthesis of Ribonuclease A*

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SUMMARY

A protected linear polypeptide of 124 amino acid residues with the sequence of bovine pancreatic ribonuclease A was synthesized by the solid phase method. The polypeptide was removed from the solid support and purified, and the four disulfide bonds were closed by air oxidation of the reduced form. The synthetic enzyme was fractionated by gel filtration and ion exchange chromatography, and the material that eluted at the same position as natural reduced-reoxidized RNase A was isolated. The product was further purified by incubation with trypsin and removal of the enzymically degraded components. A fractional precipitation with (NH₄)₂SO₄ gave a purified synthetic RNase A with a specific activity of 78%.

The synthetic RNase A was indistinguishable from natural bovine pancreatic RNase A by gel filtration on Sephadex G-75, by chromatography on carboxymethylcellulose, and by electrophoresis. Amino acid analyses, peptide maps of trypic digests, and the Michaelis constant agreed well with those of the natural enzyme. The synthetic enzyme showed the high substrate specificity to be expected of RNase A. It was highly active against yeast RNA and 2',3'-cyclic cytidine phosphate and was completely inactive toward DNA, 2',3'-cyclic guanosine phosphate, and the dinucleotides 5'-3'-guanylyl)-cytidylic acid and 5'-3'-adenylyl)-cytidylic acid.

During the synthesis, samples were removed after 99 and 104 residues had been coupled and the corresponding polypeptides, des-(21-25)S-protein and S-protein, were isolated. They were then reduced and reoxidized in the presence of NH₄ terminal residues of S-protein are not required for the protein to oxidize and fold in the presence of S-peptide to give an active enzyme.

The chemical synthesis of bovine pancreatic ribonuclease A was undertaken in an effort to provide a new approach to studies on the relation of structure to function in enzymes. The accessibility of synthetic enzymes, and of derivatives of them, can lead to information about binding sites, catalytic sites, cross-linking, and folding that is not readily attainable by other methods. Ribonuclease A was selected because it was a relatively small, stable, crystalline protein which had been studied in great detail in many laboratories (1-3). Both its linear amino acid sequence (4-8) and its crystallographic structure (9, 10) were known. Furthermore, it had been established (11-13) that the denatured, reduced chain could be reoxidized and refolded into a structure possessing full enzymic activity. Thus, the chemical synthesis of the linear sequence of ribonuclease could be expected to lead to a total synthesis of the enzyme.

The solid phase method (14, 15) was chosen for the synthesis because of the advantages in yield, speed, simplicity, and manpower requirements. The synthetic enzyme possessed high specific activity and closely resembled the natural enzyme. A preliminary account of our synthesis of ribonuclease A was published last year (16) and simultaneously a synthesis of ribonuclease S was reported by Hirschmann et al. (17). The details of our original synthesis together with some further findings are described here.

MATERIALS AND METHODS

Reagents

Boc-amino acids1 were purchased from Schwarz BioResearch and Cyclic Chemical Company. Their thin layer chromatographic behavior, melting points, and optical rotations were checked prior to use. The 1% cross-linked styrene-divinylbenzene resin (100 to 200 mesh beads) was a gift from the Dow Chemical Company. Natural RNase A (RAF, phosphate-free) for comparative studies and yeast RNA were obtained from Worthington. Natural S-peptide and natural S-protein were a gift from Dr. Erhard Gross, National Institutes of Health, Bethesda, Maryland. All chemicals used were analytical reagents. Urea was deionized over AG 501-X8 (D) mixed bed resin. The optical densities of column eluents were recorded on an ultraviolet analyzer (model UA-2, ISCO, Inc.) at 254 nm, then the appropriate fractions were read at 260 nm in the Beckman spectrophotometer. When indicated, the protein concentration of a solution was determined by the ninhydrin reaction (18). Free peptides were hydrolyzed in 0.1 N HCl in sealed, evacuated tubes for 24 hours at 110°. Peptide resins (10 mg) were suspended in a mixture of 2 ml of 12 N HCl, 1 ml of acetic acid, and 1 ml of phenol, and

1 The nomenclature and symbols follow the Tentative Rules of the IUPAC-IUB Commission on Biochemical Nomenclature (J. Biol. Chem., 241, 2401 (1966) and 242, 555 (1967)).

2 First used by Dr. M. Zaoral during his tenure as a Visiting Investigator in this laboratory.
the tubes were cooled, evacuated, and sealed. They were heated at 110° for 24 hours, and then cooled before opening. The resin was removed by filtration and washing, and the filtrate was extracted with chloroform. It was evaporated to dryness and made to volume for amino acid analysis.

To avoid problems of determining dry weights of proteins all quantities of RNase samples and standards were determined from amino acid analyses. The mean of the molar ratios of all of the accurately measurable amino acids in an acid hydrolysate was used to calculate the concentration of the protein.

Yields for solid phase peptide synthesis were calculated from the amount of the first amino acid attached to the resin. These values were based on the limiting reactant rather than on the excesses of amino acid reagents that were used in the synthesis but, if desired, can be expressed in that way from the recorded excesses.

Ribonuclease A Assays

When yeast RNA was the substrate RNase A activities were determined either by the Kunitz method (19) from the initial velocities of the hypochromic shift at 300 nm or by spectrophotometric measurement at 260 nm of the acid-soluble oligonucleotides of the digestion mixtures (20, 21). The unknown samples were compared with natural RNase A standard solutions whose concentrations were 2, 1.5, and 1 μg/ml of assay mixture for the Kunitz method, and 0.0525, 0.0350, and 0.0175 μg per ml of assay mixture for microspectrophotometric measurement.

The unknown samples were diluted with 25 ml of 50% acetic acid. The chloride ion was displaced by the addition of 5 ml of concentrated nitric acid and was determined quantitatively by a Volhard titration. The chloride was displaced by the addition of 5 ml of concentrated nitric acid and was determined quantitatively by a Volhard titration. The chloromethyl resin contained 1.19 mmole of chloride per g.

N\textsuperscript{α}-Boc-N\textsuperscript{α}-Boc-histidine, 0.84; N\textsuperscript{α}-Boc-N\textsuperscript{α}-Boc-histidine, 0.68.

EXPERIMENTAL PROCEDURE AND RESULTS

Solid Phase Synthesis of Protected Linear 124-Amino Acid Residue Polyptide Chain of Ribonuclease A

The general procedures of the automated solid phase method were followed (22, 23), but with several changes in detail. The synthesis began with the attachment of the carboxyl terminal amino acid, valine, to the resin support and continued with the stepwise addition of protected amino acids until the 124-amino acid chain was assembled on the resin (Fig. 1). The peptide was then cleaved from the resin, deprotected, purified, and isolated as described below.

Polystyrene-1%-divinylbenzene beads (100 to 200 mesh) were characterized by swelling experiments in two ways. Microscopic examination showed the average diameter of the dry beads to be 160 ± 20 μ (range 100 to 200 μ). After swelling in dry dimethylformamide the average diameter was 230 ± 16 μ, and in CH\textsubscript{2}Cl\textsubscript{2} it was 290 ± 15 μ. The volume of 1.00 g of packed dry beads was 1.70 ml. After swelling in dimethylformamide the volume was 5.20 ml, and in CH\textsubscript{2}Cl\textsubscript{2} it was 8.60 ml.

The styrene-1%-divinylbenzene beads (50 g) were thoroughly washed (14) and then were chloromethylated in 295 ml of re-distilled chloromethylmethyl ether at 0° by the dropwise addition of 5 ml of stannic chloride dissolved in 50 ml of chloromethylmethyl ether, with stirring for 30 min, and were worked up as described previously (14). A 250-mg sample was quaternized by heating in 2.5 ml of pyridine for 1 hour at 100°, and the suspension was diluted with 20 ml of 90% acetic acid. The volume was 1.00 g of packed dry beads was 1.70 ml. After swelling in dimethylformamide the volume was 5.20 ml, and in CH\textsubscript{2}Cl\textsubscript{2} it was 8.60 ml.

The chloromethyl resin (10 g) was esterified by refluxing in 75 ml of ethyl acetate with 808 mg (4.0 mmoles) of Boc-L-valine and 0.50 ml of triethylamine (3.6 mmoles) for 50 hours. The mixture was filtered, and after partial evaporation and addition of water the filtrate was lyophilized. The resulting white, amorphous powder was dried in a vacuum at 80° over KOH pellets. Yield: 1.25 g (84%).

C\textsubscript{11}H\textsubscript{17}N\textsubscript{3}O\textsubscript{4}

Calculated: N 16.46, C 51.54, H 6.68

Found: N 16.24, C 51.54, H 6.68

\( R_f \) values from thin layer chromatography on silica gel (Solvent: 1-butanol-acetic acid-water-pyridine, 30:6:24:20); N\textsuperscript{α}-Boc-N\textsuperscript{α}-Boc-histidine, 0.84; N\textsuperscript{α}-Boc-histidine, 0.68.

The abbreviation used is: TFA, trifluoroacetic acid.
were removed after the peptide chain reached 36, 75, 99, and 104 residues in this peptide. Following each deprotection step the entire stable and, as will be seen, this reagent also caused a complete deprotection even of the sterically hindered amino acid residues in this peptide. For an active ester cycle the addition was replaced by a dimethylformamide wash, and the CH₂Cl₂ wash before the Boc-amino acid p-nitrophenyl ester was omitted. Boc-Arg(NO₂) and Boc-His were first dissolved in 4.8 ml of dimethylformamide and then diluted with 2.2 ml of CH₂Cl₂ before adding to the peptide-resin. In the case of Boc-His, 5-fold excesses of amino acid and dicyclohexylcarbodiimide in CH₂Cl₂ were used. The reaction time for all dicyclohexylcarbodiimide couplings was 5 hours. Boc-asparagine and Boc-glutamine were coupled as p-nitrophenyl esters in dimethylformamide (25), employing a 4-fold excess of reagent and a reaction time of 10 hours.

One cycle of the synthesis (dicyclohexylcarbodiimide coupling) consisted of: CH₂Cl₂, twice for 2 min; 50% TFA-CH₂Cl₂, 2 min; 50% TFA-CH₂Cl₂, 21 min; CH₂Cl₂, five times for 2 min; CHCl₃, three times for 2 min; 10% Et₂N-CH₂Cl₂, 7 min; CHCl₃, three times for 2 min; CH₂Cl₂, three times for 2 min; Boc-amino acid, 7 min; dicyclohexylcarbodiimide, 300 min; CH₂Cl₂, twice for 2 min; EtOH, three times for 2 min. For an active ester cycle the CH₂Cl₂ wash before the Boc-amino acid p-nitrophenyl ester addition was replaced by a dimethylformamide wash, and the dicyclohexylcarbodiimide step was omitted.

The course of the synthesis was followed by removing 10-mg samples at intervals of 6 to 10 residues during the run and determining amino acid ratios on acid hydrolysates of the peptide-resin. In addition to the small analytical samples, larger samples were removed after the peptide chain reached 36, 75, 99, and 104 residues so that peptides representing partial sequences of ribonuclease could be isolated. A total of 1.70 g of samples, corresponding to 47% of the original 2 g of resin, was removed. The final weight of the fully protected 124-residue RNase-resin was 1.59 g. The product contained 4 NO₂-, 49 Bzl-, 10 Z-, and 4 sulfoxide groups and had a calculated molecular weight of 19,791.

Amino acid analyses showed this to contain 0.0212 mole of protein per g of protected RNase-resin, from which it could be calculated that the product contained 664 mg of protected peptide plus 926 mg of polyisoprene, or 0.0364 mole of peptide per g of polyisoprene. Therefore, the retention of peptide chain on the resin was 17%.

After the primary sequence of RNase A had been assembled it was necessary to cleave the polypeptide from the solid support, to remove protecting groups, and to purify the resulting protein. Of the several experiments that were performed, under a variety of conditions, three runs (A, B, and C) will be described here.

Cleavage of Ribonuclease A from Resin

Run A—Protected RNase-resin (200 mg) was placed in a Daiflon (polytrifluoroethylene) reaction vessel of an HF cleavage apparatus (Toho Company, Japan), dried under high vacuum, and treated according to the general procedures of Sakakibara et al. (26) and Lenard and Robinson (27). TFA (2 ml) was added, followed by 2 ml of anisole as a trap for benzyl1 and NO₂⁻ ions, and the system was evacuated again. HF was collected in a storage vessel containing CoFe, and 10 ml were redistilled into the reaction vessel which had been cooled to −78°. The temperature was raised to 0° and this temperature was maintained for 60 min, after which it was allowed to rise slowly to 20° over a period of 30 min. The HF and TFA were evaporated with a water pump, and the final traces of HF and most of the anisole were removed with an oil pump protected with a Dry Ice trap. Residual anisole and its derivatives were extracted with ether. The cleaved, deprotected peptide was dissolved in 15 ml of TFA and filtered from the resin. The filtrate and TFA washes were evaporated to dryness, and the solid protein residue was treated with 10 ml of 5% sodium bicarbonate in order to reverse any N → O acyl shift which might have occurred during the HF step (28). After 2 hours at pH 7.5, the solution was dialyzed in the cold against distilled water and then lyophilized.

Amino acid analysis showed 1.77 µmoles of peptide, indicating a 41% yield in the cleavage step. The over-all yield was 7% calculated from the amount of COOH-terminal valine originally esterified to the resin. This experiment was repeated several times, giving a total of 5.69 µmoles of crude cleaved peptide.

Run B—A 203-mg sample of protected RNase-resin was treated with HF as before, but without added trifluoroacetic acid. The work-up was the same as described for Run A. Amino acid analysis showed 3.03 µmoles of peptide, indicating a 70% yield in this cleavage step. The over-all yield was 12%.

Run C—A 340-mg sample of the 124-residue polypeptide-resin was cleaved from the solid support and was partially deblocked by passing a stream of hydrogen bromide gas through a suspension of the peptide-resin in a mixture of 10 ml of trifluoroacetic acid and 10 ml of CH₂Cl₂ (29) containing 2 ml of anisole. After 90 min at 25° the cleaved synthetic material was filtered, the resin was washed with 15 ml of TFA, and the combined filtrates were evaporated to dryness. The product was treated with 20 ml of 0.05 M NH₄HCO₃ buffer at pH 7.2. Lyophilization of this

A. Marglin, unpublished.
solution gave the partially protected 124-residue polypeptide. It was dried in a vacuum at 40°C for 5 hours. In order to remove the S-benzyl blocking groups from the 8 cysteinyl residues the synthetic product was treated with 10 ml of anhydrous HF in the presence of 1 ml of anisole, as described for Run A, and the NH₂HCO₃ treatment was repeated. Amino acid analysis of the hydrolyzed product showed 2.96 μmoles, indicating a cleavage yield of 41%. The over-all yield was 77%.

**Purification of Synthetic Ribonuclease A**

A. Ion Exchange Chromatography and Gel Filtration of S-Sulfonate of RNase A Cleaved by HF in Presence of Trifluoroacetic Acid

Oxidative Sulfitolysis (80, 31) of Liberated Polypeptide—The crude synthetic product (3 μmoles) was dissolved in 8 ml of 0.2 M phosphate buffer, 8 M in urea, pH 7.5. Then 150 mg of sodium sulfite (100 fold excess per disulfide bond) and 150 mg of sodium tetrathionate were added alternately in small portions during a period of 15 min. The reaction was allowed to proceed at room temperature for 15 hours. After a 3-hour dialysis to remove salts and urea, the solution was freeze-dried, yielding 40 mg of a white, fluffy material.

Paper electrophoresis was performed with a small sample of the crude RNase(S-SO₃)₂ in 2.4 M formic acid, 4 M in urea, pH 2.25, for 120 min at 1000 volts (Fig. 2). It showed a major Pauly positive spot which moved toward the cathode with the same mobility (R₂₅₀ 0.28) as that of the S-sulfonate of natural RNase A, together with two minor components of neutral and negative charge. Since RNase A has 19 positive charges at pH 2.25 and RNase(S-SO₃)₂ has 11, they were both expected to move toward the cathode. In order to remove the negatively charged impurity the crude RNase(S-SO₃)₂ was dissolved in 1 ml of 2.4 M formic acid, 4 M in urea, and applied on a Dowex 1-X2 column (2.1 x 15 cm) which was eluted with the same solvent. The anionic fraction was retained, the neutral and the cationic material emerged from the column as a single sharp peak. The protein-containing fractions were pooled (8.5 ml). Most of the urea was removed through a 2-hour dialysis. After lyophilization, 2 ml of water were added, the solution was adjusted to pH 5.2, and was then submitted to gel filtration on a Sephadex G-75 column (2.1 x 45.5 cm) by elution with 0.05 M formic acid, 4 M in urea, pH 2.25, 1000 volts, 120 min. The spots were detected by the Pauly spray. Crude RNase(S-SO₃)₂ was purified by ion exchange chromatography on Dowex 1-x2 and gel filtration on Sephadex G-75. The synthetic RNase A used for the electrophoresis was a sample of the IRC-50-fractionated material from Run A, Peak I. R₂₅₀ of natural and purified synthetic RNase(S-SO₃)₂ was 0.28, that of natural and synthetic RNase A was 0.38.

Three peaks. After lyophilization of the solutions the components were obtained in the following yields: Peak I (V₉ = 34 to 47 ml), 5.5 mg (15%); Peak II (V₉ = 76 to 92 ml), 16.5 mg (45%); Peak III (V₉ = 93 to 140 ml), 11 mg (30%). Peak I was sharp and appeared at the void volume of the column. It consisted of interchain disulfide-linked aggregates of RNase A, which were derived by oxidation from the freshly cleaved mixture of reduced polypeptides during incubation with 5% sodium bicarbonate solution and during the following lyophilization. These steps did not provide the necessary conditions (11-13) for an exclusive formation of intramolecular disulfide bonds. Peak II, emerging from the column between 76 ml and 92 ml, agreed well with natural RNase A with respect to elution volume (V₉ of natural RNase A, 76 to 90 ml) and electrophoretic mobility. The amino acid analysis of the material from Peak II, Run B, is given in Table I. The specific activity of this preparation was 5% when assayed against RNA. Peak III comprised a mixture of peptides with a lower molecular weight than that of the natural enzyme. These RNase A fragments were presumably obtained by incomplete coupling reactions during the assembly of the amino acid sequence of RNase A.

The protein aggregates under Peak I were reduced and then carefully reoxidized in dilute solution (0.02 mg per ml) under the conditions described below for RNase(S-SO₃)₂. The re-formed product then separated on Sephadex G-75 into two main components whose yields were 3.0 mg and 1.6 mg, respectively. Their positions agreed with those of Peaks II and III of the first separation on this column. This is explained by the conversion of the intermolecular disulfide bridges to intramolecular links through reduction and reoxidation under the proper conditions. Figures for this run are not shown because the patterns closely resembled those obtained for Run C.

B. Sephadex G-75 Gel Filtration of Ribonuclease A Cleaved in HBr and Deprotected with HF

A 1.46-μmole sample of the RNase A, derived from HBr and HF treatment, was dissolved in 1 ml of 0.05 M NH₄HCO₃ buffer. This solution was applied to a Sephadex G-75 column (2.1 x 45.5 cm) and was eluted with the same buffer. The elution diagram obtained by measuring the optical density of the fractions at 280 nm showed three peaks (Fig. 3A). The contents of the three peaks were isolated through lyophilization with the following yields: Peak I, 5.7 mg (29%); Peak II, 7.5 mg (39%);
Reduction and reoxidation of purified synthetic RNase(S-SO₄⁻)₈ from Run A was dissolved in 1 ml of 8 M urea. Then 10 μl of β-mercaptoethanol were added and the mixture was flushed with nitrogen for 15 min. After adjusting to pH 8.2 with 5% methylamine, the reduction was allowed to proceed for 20 hours at room temperature. At the end of this time, 0.3 ml of glacial acetic acid was added. After adjusting to pH 8.3 at the glass electrode with 4 N HCl, the protein was allowed to air-oxidize in a 400-ml beaker for 24 hours at room temperature to form the four disulfide bonds. An aliquot of the solution was dialyzed and assayed for RNase A activity. The crude preparation had a specific activity of 1.1 to 1.7%. In order to isolate the synthetic enzyme, the reoxidation mixture was acidified to pH 4 with 4 N HCl and freeze-dried. The synthetic protein was separated from salts on the Sephadex G-75 column in 0.1 M acetic acid. Lyophilization of the protein fraction gave the salt-free synthetic enzyme.

### Fractionation of Synthetic Enzyme on IRC-50

Further purification of the synthetic RNase A preparations was achieved through cation exchange chromatography on IRC-50 (32). The elution pattern obtained depended strongly on the previous work-up conditions for the protein.

**Run A**—When the protein chain was removed from the resin with anhydrous HF in the presence of trifluoroacetic acid, converted to the S-sulfonate, reduced with mercaptoethanol, and finally air-oxidized to form the four disulfide bridges of RNase A, the fractionation on an IRC-50 column (1 × 25 cm) with 0.2 M phosphate buffer, pH 6.47 (33), gave five peaks by ultraviolet analysis. In a typical experiment, 4.5 mg (0.33 µmole) of synthetic enzyme in 0.25 ml of the phosphate buffer were applied to the column and eluted in the same buffer. The protein content of the fractions (Fig. 4A) were determined by quantitative amino acid analysis. Aliquots of each of the pooled fractions were separated from salts on the Sephadex G-75 column in 0.1 M acetic acid. Lyophilization of the protein fraction gave the salt-free synthetic enzyme.
were assayed for enzymic activity against yeast RNA as substrate. The protein from Peak I had a specific activity of approximately 13% by the Kunitz assay and also by the Anfinsen assay (Fig. 5). The protein from Peak II showed 9.7% activity, which was partially due to the incomplete separation of Peak I from Peak II. The other three components were inactive. The fractions were lyophilized and phosphate was removed by gel filtration on Sephadex G-25 in 0.1 M acetic acid.

Yields: Peak I, 1.8 mg (40%); Peak II, 1.0 mg (22%); Peak III, 0.6 mg (13%); Peak IV, trace; Peak V, trace.

A typical amino acid analysis of material from the IRC-50 Peak I, Run A is shown in Table I.

On paper electrophoresis at pH 2.25 this synthetic RNase A was indistinguishable from the native enzyme (Rg, 0.58, Fig. 2).

Run B—When the polypeptide was removed from the resin with anhydrous HF in the absence of trifluoroacetic acid, purified by gel filtration on Sephadex G-75, reduced and reoxidized, and then fractionated (5 mg) on an IRC-50 column (1 × 25 cm) with 0.2 M phosphate buffer, pH 6.47, only three peaks were observed (Fig. 4B). The fractions from each of the three peaks were combined, lyophilized, and desalted on Sephadex G-25 in 0.1 M acetic acid. Yields: Peak I, 3 mg (60%); Peak II, 0.45 mg (9%); Peak III, 0.3 mg (6%). RNase A activity was found only in Peak I. The isolated protein from Peak I had a specific activity of 13%.

Run C—When the polypeptide was cleaved and fully deprotected with HBr and HF in a two-step reaction, partially purified on Sephadex G-75, reduced, reoxidized, and then submitted to cation exchange chromatography on IRC-50, only one peak was obtained. The synthetic enzyme (5 mg) in 0.25 ml of 0.2 M phosphate buffer, pH 6.47, was applied on an IRC-50 column (1.4 × 47.5 cm) and eluted in the same buffer (Fig. 4C). All of the eluted fractions were removed for alkaline hydrolysis followed by the ninhydrin reaction to estimate the protein concentration. The specific activity, measured as before with RNA as substrate, was 16%. The fractions eluted between 32 ml and 42 ml (Peak I) were pooled, lyophilized, and desalted on Sephadex G-25 in 0.1 M acetic acid. Yield: 3.6 mg (72%).

Fractionation of Synthetic Enzyme on CM-cellulose

A column (1.1 × 23.5 cm) of CM-cellulose was poured and equilibrated with a pH 6.0 sodium phosphate buffer, 0.01 M in Na⁺ (11). The samples were applied in the 0.01 M buffer and washed with a total of 60 ml of the same buffer. A gradient was then started with pH 7.5 sodium phosphate buffer, 0.1 M in Na⁺. The eluate was monitored at 254 nm and with the nin-
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Fig. 5. RNase A assay using yeast RNA as substrate. The assay was performed according to the procedure of Anfinsen et al. (20) as modified by Egami, Takahashi, and Uchida (21); 3-mg samples of RNA in 1 ml of 0.05 M Tris-HCl buffer, pH 7.5, were incubated with different amounts of natural and synthetic RNase A (IRC-50 Peak I, Run A) for 15 min at 37°C. The optical densities at 260 nm of the acid-soluble fractions of the digestion mixtures were plotted against the RNase A concentrations of the samples. From the ratio of the slopes of the two curves (0.53 for the synthetic, 4.0 for the natural enzyme) a specific RNase A activity of 13% was calculated.

Fig. 6. Ion exchange chromatography of synthetic, reduced-reoxidized natural, and untreated natural RNase A on CM-cellulose. After the samples had been applied, the column (1.1 X 23.5 cm) was eluted with 60 ml of 0.01 M phosphate buffer, pH 6.0. Then gradient elution toward 0.1 M phosphate buffer, pH 7.5, was started. A, 5.0 mg of untreated natural RNase A; B, 1.9 mg of reduced-reoxidized natural RNase A; C, 1.9 mg of synthetic RNase A (IRF-HF-treated, fractionated on Sephadex G-75, reduced, and reoxidized), then 0.5-ml (Chromatograms A) and 1.0-ml aliquots (Chromatograms B and C) of each fraction of the eluate from the column were submitted to alkaline hydrolysis. The hydrolysates were reacted with ninhydrin and the optical densities at 570 nm were measured spectrophotometrically.
TABLE II

Stability of natural RNase A toward trypsin

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<th>Ratio of trypsin to RNase A (w/w)</th>
<th>Recovery of RNase A activity</th>
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<tr>
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<td>1:4</td>
<td>80%</td>
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<td>1:2</td>
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- Samples of natural RNase A (5 mg per ml) were incubated with trypsin in 0.2 M ammonium bicarbonate buffer at pH 7.7 for 13 hours at room temperature.

The mixture was placed in the cold room. After 2 days, some amorphous precipitate had formed. It was centrifuged. An aliquot of the supernatant solution was assayed again by the Kunitz method. The activity against RNA of 0.25, 0.75, and 2.5 µg per ml of synthetic RNase A was compared with that of 1.0 µg per ml of natural enzyme. In this assay (Fig. 7) the specific activity of the purified synthetic material at the three enzyme levels was found to be 80, 75, and 80%; average specific activity, 78%. This value was confirmed by the Anfinsen assay.

The amorphous precipitate was dissolved in 0.5 ml of 0.06 M ammonium bicarbonate buffer. From quantitative amino acid analysis of an acid hydrolysate the yield of the amorphous fraction was found to be 0.21 mg. It showed a specific activity of 16%. Table III summarizes the activity data on the trypsin- and ammonium sulfate-treated synthetic RNase A preparations.

Preparation of Peptide Maps from Natural and Synthetic Ribonuclease A

Samples of natural and synthetic RNase A were oxidized with performic acid (33). The oxidized derivatives were then susceptible to trypsin digestion. Trypsin, 1 mg, was dissolved in 40 ml of 0.2 M ammonium bicarbonate buffer, pH 7.7, and 0.5 ml of this solution (containing 0.0125 mg of trypsin) was then added to 2.5 mg of oxidized RNase A. After an incubation time of 22 hours at room temperature the reaction was stopped by adjusting to pH 2. When peptide maps were not prepared immediately the samples were stored frozen.

Usually 0.2 ml of the digest (1 mg) was applied on Whatman No. 3MM chromatography paper. The mixture was resolved by high voltage electrophoresis at 2250 volts in a pH 3.5 buffer containing 2.2 ml of pyridine per liter and 22 ml of acetic acid per liter, in a LT 48A tank (Savant Instrument, Inc.) in the first dimension and descending paper chromatography in phenol-1-butanol-acetic acid-water (3:3:2:4) in the second dimension. The air-dried chromatograms were developed with the ninhydrin-cadmium acetate spray (34). In each experiment lysine was run as a standard.
pared to that of lysine.

The chromatograms were sprayed with the ninhydrin-cadmium acetate reagent. The positions of the tryptic peptides were compared to that of lysine ($R_{Lys} = 1$).

are shown in Fig. 8. The peptide maps were prepared two more times each and were found to be reproducible.

The relative positions of the 14 expected (18) ninyhydrin-positive spots on the two chromatograms agreed quite well, but some of the corresponding peptide pairs gave spots of different shape and size. In addition, there was one spot on the peptide map of the synthetic material near the position of lysine which could not be seen in the natural RNase control. It was not identified, however, and its origin is not known.

Enzymic Digestion of Synthetic Ribonuclease A with Papain and Aminopeptidase M

In order to establish its optical purity the synthetic enzyme (IRC-50 Peak I, Run A) was treated with papain followed by aminopeptidase M. After natural RNase A was shown to be digested by this procedure, 0.14 mg of synthetic material (10 pmoles) was dissolved in 95 μl of 0.05 M ammonium acetate buffer, pH 5.3, then 5 μl of a 3% solution of mercaptoethanol and a suspension of 10 μg of papain (15 units per mg, Worthington) in 1 μl of 0.06 M sodium acetate, pH 4.5, were added. The mixture was incubated at 37° for 2 hours, at which time the papain was inactivated with 2 drops of glacial acetic acid and the reaction was continued for 3 more hours at 37°. Then 0.3-mg portion of aminopeptidase M in 15 μl of water was added. After incubation for 3 hours at 37° the reactions were stopped by adding 50 μl of 0.2 M mercuric chloride solution to each sample. Then the digested mixtures were resolved on IRA-400 and the optical densities of the column effluents were recorded at 271 nm. Peak a, excess undigested 2',3'-cyclic cytidine phosphate; Peak b, 3'-cytidine phosphate hydrolysis product. For synthetic RNase A an average activity of 24.5% was calculated when the area under Peak b was compared with that of the natural RNase A control.

RNase A or during the various work-up procedures. It also revealed that 79% of the methionine sulfoxide residues had been reconverted to methionine residues during the mercaptoethanol reduction of the synthetic RNase($\text{S-SO}_2^-$). The remainder was recovered as methionine sulfone. The Boc-methionine sulfoxide (35) used in the synthesis of the polypeptide chain of RNase A was chromatographically pure and did not contain any detectable amount of the sulfone derivative. Since air was not excluded and no special precautions against peroxide formation were taken it is assumed that the sulfone arose by a gradual oxidation during the synthesis.

Substrate Specificity of Synthetic Ribonuclease A

Since RNase A has a very high specificity for the cleavage of the 5'-ester bond of 3',5'-phosphodiester bonds in which a pyrimidine ribonucleotide provides the 3'-ester, it was necessary to demonstrate that the synthetic enzyme possessed the same substrate specificity. It should split RNA, pyrimidine oligoribonucleotides, and pyrimidine nucleoside 2',3'-cyclic phosphates, but not DNA, deoxyribonucleotides, purine ribonucleotides, or purine...
Fig. 10. DNase assay with synthetic RNase A. The DNase assay, using calf thymus DNA as substrate, followed the procedure of Kunitz (37). Three samples of DNA (0.2 mg each) in 5 ml of solution, pH 5, containing 0.005 M Mg++, were mixed at 25° with 0.005 mg of DNase I, 0.2 mg or 0.02 mg of synthetic RNase A in 1 ml of water. The change of the optical density was followed spectrophotometrically at 260 nm for 10 min. The absorbance of the samples containing synthetic RNase was recorded up to 2 hours and showed no increase, demonstrating that no reaction occurred between synthetic RNase A and DNA during that time.

cyclic phosphates. Most of the data on substrate specificity were obtained on material from IRC-50 Peak I, Run A (Fig. 4A).

RNA as Substrate—When yeast RNA was the substrate RNase A activities were determined either by the Kunitz method (19) from the initial velocities of the hypochromic shift at 300 nm or by spectrophotometric measurement at 260 nm of the acid-soluble oligonucleotides of the digestion mixtures (20, 21). The specific activities when measured by the two methods have been in good agreement. For example, the material from IRC-50 Peak 1, Run A was 13% as active as natural RNase A by the Kunitz assay and also 13% by the Anfinsen assay (Fig. 5). Depending on the work-up conditions and the extent of purification, the various synthetic RNase A samples have shown enzymic activities against RNA that have been up to 78%, as high as pure native RNase A (Fig. 7).

2',3'-Cyclic Cytidine Phosphate (C>p) as Substrate—The synthetic enzyme was assayed against C>p by the procedure of Fruchter and Crestfield (36). The C>p (1.4 mg) was incubated at 0° with 20 µg of RNase in 2 ml of Tris-NaCl buffer, pH 7.48, and after 10 min the reaction was stopped with 50 µl of 0.2 M mercuric chloride solution. The hydrolysis product (3'-cytidine phosphate) and excess substrate were separated on an IRA-400 column by elution with Tris-NaCl buffer, pH 7.48. The extent of hydrolysis was quantitatively determined by integration of the peaks recorded at 271 nm on a Zeiss PMQ II spectrophotometer. The specific activities of the synthetic samples were calculated by comparison with natural RNase A standards after correcting for the blank hydrolysis values. The synthetic enzyme from IRC-50 Peak I, Run A showed a specific activity of 24.5% (Fig. 9). The (NH₄)₂SO₄-purified preparation had a specific activity of 65% against this substrate.

DNA as Substrate—DNase activity was measured by the method developed by Kunitz (37), based upon the increase of absorption at 260 nm during the depolymerization of deoxyribonucleic acid by DNase at 25°. To four samples of the substrate each containing 0.2 mg of DNA (calf thymus, Worthington) in

Fig. 11. Assay of synthetic RNase A for RNase T₁ activity. 2',3'-Cyclic guanosine phosphate (top) and 5'-3'(guanylyl)-cytidylic acid (bottom) were used as substrates for the RNase T₁ assay. Aliquots of the digests were chromatographed on Silica Gel GP-coated thin layer plates using isopropl alcohol-water-ammonium hydroxide (70:25:5) as solvent. The spots were detected under an ultraviolet lamp. A, 10 µg each of RNase T₁ and natural and synthetic RNase A were added to solutions of 40 µg of 2',3'-cyclic guanosine phosphate (G>p) in 0.2 ml of 0.1 M ammonium bicarbonate buffer. The samples were incubated 48 hours at 25°. G>p was hydrolyzed in the presence of RNase T₁ to give 3'-guanosine phosphate (Gp), but not by the RNase A samples. B, 10 µg each of RNase T₁ and natural and synthetic RNase A were mixed with solutions of 100 µg of 5'-3'(guanylyl)cytidylic acid (GpCp) in 0.1 ml of 0.1 M ammonium bicarbonate buffer and incubated 48 hours at 25°. The substrate was hydrolyzed by RNase T₁ to give C>p as an intermediate and Gp and 3'-cytidine phosphate (Cp). The RNase A samples had no effect on this substrate.
substrate by the Kunitz method. Samples added to solutions of 0.1 mg of GpCp (39) in 0.1 ml of 0.1 M ammonium bicarbonate buffer. After an incubation time of 48 hours at 25°C, aliquots were analyzed by thin layer chromatography on silica gel plates in isopropyl alcohol-water-NH₄OH (70:25:5). The spots were detected under ultraviolet light (Fig. 11B). Under these conditions RNase T₁ completely cleaved this dinucleotide into cyclic guanylic acid, guanylic acid, and cytidylic acid, whereas the native and synthetic RNase A had no effect whatever and GpCp was recovered unchanged.

S'-(3'-Adenylyl)-adenylic Acid (ApAp) as Substrate—Finally the specificity of the synthetic enzyme for the 3',5'-phosphodiester bond adjacent to a purine ribonucleotide was confirmed by assays using ApAp (39) as substrate. RNase T₁, natural RNase A, or synthetic RNase A (10 µg each) were dissolved in solutions of 0.2 mg of ApAp in 0.2 ml of 0.1 M ammonium bicarbonate buffer and incubated for 48 hours at 25°C. Upon thin layer chromatography in the above system the sample containing either RNase T₁, natural RNase A, or synthetic RNase A had the same Rₑ as the blank (ApAp), and no spot for Ap was visible, indicating the expected resistance of this dinucleotide to all three enzymes.

**Determination of Michaelis Constant**

Initial velocities of RNA hydrolysis were measured spectrophotometrically at 300 nm by the Kunitz method. Each sample of the substrate was dissolved in 2 ml of 0.1 M sodium acetate buffer, pH 5, and then mixed with either natural or synthetic RNase A in 2 ml of water. The final volume was 4 ml. Seven different substrate concentrations were used: 3.0, 1.8, 1.2, 0.60, 0.45, 0.30, and 0.18 mg per ml. The enzyme concentrations were constant in all experiments: 2.5 µg per ml for natural and 16.7 µg per ml for synthetic RNase A (from IRC-50 Peak I, Run A). The absorbance at 300 nm was followed for 10 min. The Kₘ values, calculated from Lineweaver-Burk plots of initial velocities, were found to be 1.20 mg per ml for natural RNase A and 1.24 mg per ml for the synthetic enzyme (Fig. 12). The values that we originally reported (16) were both in error by a factor of 2 because of an incorrect dilution calculation. Edelhoch and Coleman (40) have reported a Kₘ value of 1.25 mg per ml for natural RNase A acting on yeast RNA.

**Synthesis of Ribonuclease S-peptide (RNase(1-20))**

S-peptide was synthesized by the solid phase method using the same procedures as described for the total synthesis of RNase A. In this synthesis Boc-Met was used instead of Boc-Met(O), but the other amino acid derivatives were the same as before. The eicosapeptide was cleaved from the resin with anhydrous HF in the presence of excess anisole (yield, 44%), and was purified by high performance liquid chromatography on silica gel plates in isopropyl alcohol-water-NH₄OH (70:25:5). The spots were detected under ultraviolet light (Fig. 11B). Under these conditions RNase T₁ completely hydrolyzed the substrate to guanylic acid, whereas neither natural nor synthetic RNase A had a detectable effect on the G₃₇ substrate.

S'-(3'-Guanylyl)-cytidylic Acid (GpCp) as Substrate—Natural RNase A, synthetic RNase A, and RNase T₁ (10 µg each) were added to solutions of 0.1 mg of GpCp (39) in 0.1 ml of 0.1 M ammonium bicarbonate buffer. After an incubation time of 48 hours at 25°C, aliquots were analyzed by thin layer chromatography on silica gel plates in isopropyl alcohol-water-NH₄OH (70:25:5). The spots were detected under ultraviolet light (Fig. 11B). Under these conditions RNase T₁ completely cleaved this dinucleotide into cyclic guanylic acid, guanylic acid, and cytidylic acid, whereas the native and synthetic RNase A had no effect whatever and GpCp was recovered unchanged.

**Preparation of Synthetic S-protein and Des-(21-25)S-protein and Their Combination with Natural or Synthetic S-peptide to Give Ribonuclease S and Des-(21-25)ribonuclease S**

During the course of the RNase A synthesis, samples (213 and 355 mg, respectively) were removed at intermediate stages containing 99 and 104 amino acid residues. They were dried in a vacuum and stored for later work-up.

**Cleavage from Resin and Deprotection of Side Chains—** A 213 mg sample of dried RNase (26-124)-resin, containing 99 amino acid
umes on Sephadex G-75 of samples of the synthetic S-protein. The yields obtained were 8.1 mg for the synthetic S-protein and 6.5 mg for the synthetic des-(21-25)S-protein, and rechromatographed on the Sephadex G-75 column. A slower moving peak consisting of a mixture of smaller peptides was also obtained in addition to the main peak. Consisting of disulfide-linked aggregates was observed and a due peptides were very similar to those obtained with crude synthetic RNase A (Fig. 3). In each case a polymer fraction patterns of the elution curves for the synthetic 99- and 104-residues were applied on a Sephadex G-75 column (2.1 x 46.5 cm). The buffer was 0.05 M ammonium bicarbonate. The lyophilized polypeptides were each digested and deprotected peptide was dialyzed and lyophilized. The 104-amino acid residue peptide was removed from the resin under the main peaks were combined, lyophilized, and rechromatographed on the Sephadex G-75 column (2.1 x 46.5 cm). The buffer was 0.05 M ammonium bicarbonate. The general fractions were pooled and diluted with distilled water to 50 ml in 8 M urea. The isolated, reduced proteins were then reoxidized in the presence of S-peptide in dilute solution (0.028 mg per ml) at pH 8.25 and room temperature for 20 hours to give RNase S and des-(21-25)RNase S.

The partially purified products were then submitted to paper electrophoresis in 2.4 M formic acid, 4 M in urea, at pH 2.25 together with natural S-protein, natural RNase A, and histidine as a control (Fig. 14). The Rf values were: synthetic S-protein, 0.57; natural S-protein, 0.57; synthetic des-(21-25)S-protein, 0.58; and natural RNase A, 0.54. Amino acid analyses of natural and synthetic S-protein and synthetic des-(21-25)S-protein are given in Table I and show satisfactory agreement when the latter is corrected for the Asp (1), Tyr (1), and Ser (3) residues that were omitted in the synthesis.

Combination of S-peptide with S-protein or des-(21-25)S-protein

The protein components were reduced with β-mercaptoethanol in 8 M urea. The isolated, reduced proteins were then reoxidized in the presence of S-peptide in dilute solution (0.028 mg per ml) at pH 8.25 and room temperature for 20 hours to give RNase S and des-(21-25)RNase S.

<table>
<thead>
<tr>
<th>Components</th>
<th>Mole ratio</th>
<th>RNase A activity</th>
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<tr>
<td>Natural S-protein + natural S-peptide</td>
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<td>37</td>
</tr>
<tr>
<td>Synthetic S-protein + natural S-peptide</td>
<td>1:2</td>
<td>5</td>
</tr>
<tr>
<td>Synthetic des-(21-25)S-protein + natural S-peptide</td>
<td>1:2</td>
<td>9</td>
</tr>
</tbody>
</table>

and synthetic des-(21-25)S-protein are compared with that of natural S-protein in Fig. 13.

The partially purified products were then submitted to paper electrophoresis in 2.4 M formic acid, 4 M in urea, at pH 2.25 together with natural S-protein, natural RNase A, and histidine as a control (Fig. 14). The Rf, values were: synthetic S-protein, 0.57; natural S-protein, 0.57; synthetic des-(21-25)S-protein, 0.58; and natural RNase A, 0.54. Amino acid analyses of natural and synthetic S-protein and synthetic des-(21-25)S-protein are given in Table I and show satisfactory agreement when the latter is corrected for the Asp (1), Tyr (1), and Ser (3) residues that were omitted in the synthesis.

Generation of Ribonuclease A Activity from Synthetic S-protein and Synthetic des-(21-25)S-protein upon Addition of Natural or Synthetic S-peptide—First, the synthetic proteins were unfolded by reduction. Synthetic S-protein or des-(21-25)S-protein (1.0 mg) was dissolved in 1 ml of 8 M urea solution, 0.01 ml of mercaptoethanol was added, and the sample was flushed with nitrogen for 10 min and then adjusted to pH 8.25 with 5% methyamine. Reduction was carried out for 20 hours at room temperature. After this time the mixture was acidified with 0.3 ml of glacial acetic acid, and the reduced protein was separated from urea and excess mercaptoethanol on a Sephadex G-25 column (2.1 x 36 cm) in 0.1 M acetic acid. The protein-containing fractions were pooled and dialyzed with distilled water to 50 ml in which 0.71 g of dibasic sodium phosphate was dissolved (0.1 M). The pH of the solution was adjusted to 8.25. Then 0.4 mg of natural or synthetic S-peptide was added, and the mixture was...
reoxidized with air for 20 hours at room temperature to allow the chains to refold and the four disulfide bonds to re-form. The protein concentration of the solution was 0.028 mg per ml and the mole ratio between the synthetic proteins and S-peptide was 1:2. As a control, natural S-protein was reduced and reoxidized in the presence of natural S-peptide.

The RNase A activities, measured spectrophotometrically by the Kunitz method (Table IV), were verified with assays by the Anfinsen method. Synthetic S-protein produced RNase S with 5% specific activity when mixed with either natural S-peptide or synthetic S-peptide. Synthetic des-(21-25)S-protein produced des-(21-25)RNase S with 9% specific activity when mixed with natural S-peptide and 7% with synthetic S-peptide. The fully synthetic RNase A (1-124) when worked up under the same conditions had 9% specific activity.

**DISCUSSION**

**Solid Phase Synthesis of Protected Linear 124-Amino Acid Residue Polypeptide Chain of Ribonuclease A**

The successful synthesis of ribonuclease A by the solid phase method required that there be enough space within the resin beads to accommodate a protected polypeptide of 19,791 molecular weight. For that reason a polystyrene resin with only 1% divinylbenzene was selected. This low cross-linking allowed good swelling in organic solvents and was the minimum that was still compatible with good physical stability of the beads. Earlier electron micrograph data (41) had indicated that under these conditions the average space available to each peptide chain probably would be large enough to accommodate the ribonuclease A molecule.

With growing chain length of the peptide the volume of the resin increased considerably, but its swelling and filtering behavior as well as its mechanical properties were not noticeably affected. During their synthesis of a cytochrome c analogue Sano and Kurihara (42) observed an extremely severe breakdown of the resin, which in the end resulted in the loss of about 80% of the polymer support from the reaction vessel. This did not occur at all in the solid phase synthesis of RNase A described here. The quality of the resin support is clearly of great importance in solid phase peptide synthesis. We have observed some variability in the properties of commercial styrene-divinylbenzene resins and of chloromethylated resins with regard to uniformity of size, mechanical stability, and chemical reactivity. It seems probable that some of the reported discrepancies between the results from various laboratories can be attributed to differences in resins.

Protecting groups for the amino acid side chains were selected which could be removed in one step together with the cleavage of the polypeptide chain from the polymer support. In general benzyl derivatives were used. However, since preliminary coupling experiments on small peptides with imidazole-protected histidine had been quite successful, Boc-histidine with an unblocked side chain (instead of Boc-\(N^\text{trim} \)-benzylhistidine) was used to incorporate the 4 residues of histidine into the amino acid sequence of RNase A. Thus, the treatment of the final product with sodium in liquid ammonia was not required and side reactions, involving desulfurization (43) and peptide bond cleavage (44, 45) which have been reported for this deprotection method, could be avoided.

Three- to five-fold excesses of amino acid reagents, and coupling times of 5 hours for dicyclohexylcarbodiimide reactions and 10 hours for active esters were chosen for this synthesis on the basis of past experience with the solid phase method. It should be noted that none of the concentrations or reaction times were optimized. We believe, however, that they were in excess of those actually required and that they were adequate to ensure nearly quantitative reactions. Since the quantity of Boc-amino acid for each coupling step was calculated from the concentration of the first valine residue, the concentration of Boc-amino acids remained constant during the run, but the concentration of peptide amino groups continually decreased due to the loss of peptide chains from the resin as the synthesis proceeded. Thus, the actual molar excess of reagent increased at each step. The effect of this drift in the ratio of the reactants on the rate and extent of the coupling reactions is not known.

In this synthesis the individual coupling steps and deprotection steps were not monitored because of the excessive time required to carry out these procedures with our present methodology. However, the desirability of applying rapid monitoring methods is clearly recognized (46), since it is of utmost importance to minimize incomplete coupling reactions and deprotection steps that would lead to deletion peptides missing amino acid residues.

The course of the synthesis was followed by sampling at intervals of 6 to 10 residues throughout the synthesis and determining amino acid ratios on acid hydrolysates of the peptide-resins. The precision of such analyses is limited, but the data served to indicate the progress of the chain growth. During the elongation of the polypeptide chain from 99 to 104 amino acid residues a sample for hydrolysis and amino acid analysis was removed after each coupling step. These data indicated plainly that even when the peptide chain had reached a length of the order of 100 residues it still reacted rapidly and in high yield with added Boc-amino acids.

Quantitative amino acid analysis of the final fully protected 124-residue polypeptide-resin indicated a yield of 17% based on the amount of COOH-terminal valine originally esterified to the polymer support, which means that an average of about 1.4% of the peptide chains were lost from the resin at each of the 123 cycles of the synthesis. This slow cleavage of the ester bond linking the growing polypeptide to the resin is attributed to the deprotection step, in which 50% (v/v) TFA-CH₂Cl₂ was used, but it was not established that the loss was uniform at every step. It must be emphasized that these data are a measure of the retention of the peptide chain on the resin but that they are not to be taken as an indication of the coupling yields. The partial losses of the benzoyloxycarbonyl group from the \(\alpha\)-amino group of lysine (47, 48) and of the \(\beta\)- and \(\gamma\)-benzyl esters of aspartic and glutamic acids during the acidic removal of the \(\alpha\)-Boc protecting groups are also recognized as possible side reactions during this synthesis. There are no definitive data showing that these reactions actually occurred, but we assume that some of the observed heterogeneity of the crude product resulted from this cause. The introduction of more acid labile amino-blocking groups like the biphenylisoproproxyloxycarbonyl (Bpoc) group (49), which can be cleaved with 0.5% (v/v) TFA-CH₂Cl₂ (50), should effectively eliminate these side reactions.

**Cleavage of Ribonuclease A from Resin**

After the synthesis of the fully protected RNase A sequence, the peptide was removed from the resin support and was de-
protected under three different conditions which have been described as Runs A, B, and C. The first experiments (Run A) on the cleavage of the protected 124-residue peptide-resin with anhydrous HF (26, 27) in TFA (51). However, the effect on the natural RNase A control of the HF TFA mixture (60 min at 0-20°C) was rather severe. There were differences in the amino acid analyses (Table I) and activities (Table V). When natural RNase A was treated with anhydrous HF in the presence of anisole, but with no TFA, the amino acid analysis was in good agreement with that of a sample not treated with HF. However, even under these conditions there probably was slight denaturation since the specific activity dropped to about 90%. 

A third sample of protected RNase-resin was cleaved and deprotected first in HBr-TFA (29) and then in HF because there was reason to believe that Tyr(BzI) might be difficult to deprotect completely in HF. Past experience had shown that the benzyl ether was cleaved completely from tyrosine in HBr-TFA. It was observed (Run C) that fewer components were actually present after such treatment, but the total number of units of RNase A activity was essentially the same as when only HF had been used.

Upon cleavage of the 124-residue peptide-resin with anhydrous HF-TFA, it was found that only 41% of the peptide chains were removed from the resin within 90 min at 0-20°C. Comparison of the analysis of cleaved peptide with that of the peptide still bound to the resin showed no significant differences in the amino acid composition. It has been found that a second treatment with HF cleaved 22% more of the peptide, making a total of 63%. However, in the original work-up the longer contact with HF was avoided because of the possible side reactions that this strong acid is known to promote (28, 52). Several precautions were taken to minimize undesired reactions which might occur in HF. The HF was dried over CoF3 (53) and redistilled onto the frozen sample. Anisole (26) was used as a trap for NO2· and HF. The HF was dried over CoF3 (53) and redistilled onto the frozen sample. Anisole (26) was used as a trap for NO2· and benzyl ether ions to prevent them from attacking tyrosine and other susceptible amino acid residues. Methionine was introduced as benzyl ether to prevent the formation of randomly oxidized monomers or of polymers. The S-sulfonates were used with such great success in all of the insulin syntheses (45, 54-56) that they were a logical choice here, too. Accordingly, RNase(−SSO3−) was prepared from the synthetic protein and from the natural enzyme by oxidative sulfotolysis with sodium sulfite and sodium tetrathionate (30, 31). The crude synthetic product showed, in addition to the major component, two minor spots on paper electrophoresis (Fig. 2). One of these was removed by chromatography on Dowex 1-X2 and the other by gel filtration on Sephadex G-50. The partly purified main fraction, which moved as a single component on paper electrophoresis, was obtained in 50% yield from the crude cleaved product. The conditions used for the S-sulfonate synthesis were clearly not optimal. Thus, a sample of natural RNase A after HF-TFA treatment, S-sulfonation, reduction, and reoxidation exhibited only 12% activity compared with 40 to 50% when the S-sulfonation step was omitted (Table V). Material from the later runs (B and C) was purified by gel filtration on Sephadex G-75 (Fig. 3). This procedure separated polymers (Peak I), randomly oxidized monomers (Peak II), and low molecular weight peptides (Peak III). The position of Peak II agreed closely with that from natural RNase A. More monomer could be obtained from the polymer peak by passing it through another reduction-reoxidation cycle.

Reduction and Reoxidation of Synthetic Ribonuclease A Preparations

It was necessary to reduce both the RNase(−SSO3−)a preparation and the randomly oxidized Sephadex G-75 fractions to the sulphydryl derivatives and then to oxidize in air, in dilute solution, to regenerate ribonuclease A, which presumably contained the proper disulfide bonds and was folded into the native threedimensional conformation. This synthesis is, of course, totally dependent on the spontaneous reoxidation and refolding of the chain to give the native structure, as originally found for natural RNase A by White (11), Anfinsen and Haber (12), and Epstein et al. (13). As a control for the experiments with synthetic enzyme it was established that full biological activity could be recovered in this laboratory when natural RNase A was reduced and reoxidized under these conditions.

There is no way at this time to direct the pairing of the proper cysteine residues or the folding of the molecule by selective chemical methods. Efforts which are being made to develop selective protection procedures for cysteine residues (57, 58)
have not yet reached the degree of refinement required for a synthesis of ribonuclease.

**Fractionation of Synthetic Enzyme on IRC-50**

This weakly acidic cation exchange resin has been used frequently for the fractionation and purification of natural RNase A (32). It was therefore chosen as a workup method in which to purify the synthetic enzyme. The number of peaks obtained when the synthetic material was fractionated on this column (Fig. 4) depended on the previous cleavage and purification procedures which had been applied to the sample.

The polypeptide (Run A) that had been cleaved in HF-TFA and carried through the S-sulfonation procedure gave rise to five peaks, whereas the material (Run D) cleaved in HF and purified by gel filtration produced three peaks, and the peptide (Run C) that had been cleaved in HBr-TFA and further deproteinized in HF before purifying by gel filtration gave rise to only a single peak. In each case the main activity was associated with Peak I, and the elution position of Peak I was exactly the same in all three runs.

In Run A, material with a specific activity of 1 to 2% was applied to the IRC-50 column and a 40% yield of product with a specific activity of 13% was obtained. The total number of units of RNase A activity recovered in this run (including that from Peak II) increased approximately 4- to 7-fold. In Run B, starting material with 5% specific activity led to a 60% yield of product with 13% specific activity (1.6-fold increase in total units). In Run C, the specific activity rose from 9 to 16% and the purification yield was 72%. Therefore, the total number of enzyme units increased after the fractionation experiments showed that, by this method, the specific activity of the synthetic RNase A could be raised to between 13 and 16%, regardless of whether the crude material applied to the column had an activity of 9% or only 1 to 2%. The fact that the total number of enzyme units increased after the fractionation suggests that inhibitory materials which had arisen during the synthesis and work-up procedures were being removed by the ion exchange column.

During these fractionation experiments on IRC-50, it was found that the elution volume of natural reduced-reoxidized RNase A agreed well with that of the synthetic enzyme, but that the position of both differed from that of untreated, pure natural RNase A on this ion exchange column. Fig. 4 (A and B) shows that despite different work-up conditions the two samples of synthetic RNase A both eluted from a short IRC-50 column at a volume of 12 ml (maximum of peaks). Reduced reoxidized natural RNase A had the same chromatographic behavior whereas the peak of untreated natural enzyme did not appear until 18 ml. On a longer IRC-50 column both reduced-reoxidized natural RNase A and synthetic RNase A (Run C, HBr + HF, cleaved, fractionated on Sephadex G-75, reduced, reoxidized) emerged between 31 and 45 ml with the peak at 37 ml and were indistinguishable from one another (Fig. 4, C and D). Again, pure untreated natural RNase A eluted later. It emerged between 47 and 62 ml with the peak at 55 ml. A mixture of reduced-reoxidized natural RNase A and untreated natural RNase A separated into two fully active peaks at 37 and 55 ml, respectively.

It has become evident that very small differences in the reduction and reoxidation conditions for natural RNase A can give rise to variable proportions of the two peaks at 37 and 55 ml on IRC-50 chromatograms. Whereas the earlier experiments gave only the 37-ml peak, later runs have given reconstituted natural RNase A containing the two peaks in a ratio as high as 1:2. Although IRC-50 columns have been used many times for the chromatographic purification of native RNase A, there does not appear to be a report in the literature on the chromatography of reduced-reoxidized RNase A on IRC-50. It is not clear, therefore, whether the reduced-reoxidized natural RNase A prepared in other laboratories would also have been distinguishable from the native enzyme by chromatography in this system. It was also shown by Weber et al. (59) that reoxidation of reduced natural S-protein in the presence of S-peptide at pH 8 gave rise to more than one component on a Sephadex CG-50 column, whereas reconstitution at pH 6.5 gave active material that chromatographed largely as a single component.

In contrast, reduced-reoxidized natural and synthetic RNase A were indistinguishable from each other and from pure native RNase A upon chromatography on CM-cellulose (Fig. 6). This is the chromatographic system that was used in the past to demonstrate the similarity between native and refolded RNase A (11). By this criterion our synthetic RNase A was identical with the natural enzyme.

**Further Purification of Synthetic Ribonuclease A by Treatment with Trypsin and by Fractional Precipitation with Ammonium Sulfate**

RNase A is known to be very resistant to tryptic digestion (60), whereas S-protein, RNase S, and denatured RNase A (61, 62) are susceptible to tryptic hydrolysis. This meant for the synthetic enzyme that molecules with a conformation which resembled closely that of native RNase A should resist tryptic attack whereas impurities, even with the same charge and molecular weight, but with different tertiary structures than that of natural RNase A might be digested by trypsin. The degradation products then would be easily separable from the intact active enzyme by gel filtration. Therefore, if the synthetic RNase A that showed a low specific activity were a mixture of a small amount of fully active enzyme and a large amount of inert protein having an incorrect primary and tertiary structure the improperly folded chains would be expected to be more susceptible to cleavage by trypsin than those chains with the native conformation of biologically active RNase A, and the specific activity of the enzyme should increase. If, on the other hand, the synthetic enzyme were a mixture of proteins each of which had a low specific activity due to deviations from the native species in amino acid sequence and three-dimensional structure, it seemed likely that trypsin would destroy virtually all the enzymic activity in the sample. If low activity were caused by the binding of a peptide inhibitor to the active site of the synthetic RNase A, trypsin might digest the peptide chain of the inhibitor and thus reactivate the enzyme.

When a sample of synthetic enzyme with about 8% specific activity was incubated with trypsin, then acidified and fractionated on Sephadex G-50 two peaks were observed. The position of Peak T-I agreed closely with that of untreated natural RNase A, whereas Peak T-II appeared to be a mixture of smaller peptides. The protein in Peak T-I was found to have a specific activity of 61%.

The amount of synthetic material digested by trypsin and removed by separation on the Sephadex column was only 25% of the starting material. This, together with the very appreciable 7.6-fold increase in the specific activity, means that the
Evidence for Chemical and Physical Purity of Synthetic Enzyme and for Its Similarity to Native Ribonuclease A

The various data recorded and discussed here generally support the view that our synthetic protein is reasonably homogeneous and that it bears a close resemblance to the natural molecule. However, a rigorous proof of purity of a synthetic protein is difficult and the use of total synthesis to prove the structure of a protein in the classic organic chemical sense becomes very difficult indeed. One can establish certain identities, but always within the limits of the particular technique being applied. We do not suggest that our work, in its present state of development, constitutes a structure proof of ribonuclease A, only that the synthetic molecule bears a close chemical and physical resemblance to the natural protein and that it is a true enzyme with a specific activity not far from that of native ribonuclease A.

The chemical and physical comparisons were based on amino acid analyses, enzyme digestions, antibody neutralization, peptide maps, paper electrophoresis, gel filtration, and ion exchange chromatography.

The purified synthetic enzyme had the over-all amino acid composition (Table I) expected of RNase A with only minor deviations from that of the untreated natural control. Enzymic digestion, first by papain and then by aminopeptidase M, completely degraded the synthetic molecule to free amino acids. This

### Table VI

<table>
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<tr>
<th>Stage</th>
<th>Amount of material carried through Run B</th>
<th>Yield calculated from 2 g of resin</th>
<th>Specific activity</th>
<th>Total RNase A activity</th>
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<tr>
<td></td>
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<td>mg</td>
<td>%</td>
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</tr>
<tr>
<td>Trypsin-resistant RNase A (Peak T-I)</td>
<td>0.69</td>
<td>0.62</td>
<td>356</td>
<td>4.4</td>
</tr>
<tr>
<td>Purified RNase A (NH₄)₂SO₄-soluble</td>
<td>0.41</td>
<td>169</td>
<td>2.9</td>
<td>78</td>
</tr>
</tbody>
</table>

* This is the weight of protein or protein-resin that was actually carried through Run B. The number on the right is the amount used for each step and the number at the left below is the yield of that step. Thus, 200 mg of protected RNase-resin were used for the HF cleavage in Run B and it gave 41.5 mg of crude cleaved protein, 37 mg of which were used for the Sephadex G-75 fractionation.
* This is the percentage of activity relative to pure native RNase A.
* The weight of pure RNase A required to give the activity observed in the synthetic preparations.
indicated that no major racemization had occurred during the synthesis or the various work-up procedures and is in agreement with the accumulated data from several laboratories (15) that the usual procedures of solid phase synthesis do not cause significant racemization. Although the synthetic RNase A was resistant to trypsin it was readily digested after performic acid oxidation (18, 23) of the 4 cysteine residues to cysteic acid residues. Peptide maps of the digest showed, as expected, 14 spots that corresponded quite well with the positions of the peptides derived from natural RNase A, and a small additional spot near the position of lysine (Fig. 8). Since the separated peptides were not further identified there is no evidence that the corresponding spots were identical. However, major differences in protein composition would have been expected to give rise to quite different peptide patterns. Peptides arising from small amounts of chains with different sequences would probably have gone undetected.

The purified synthetic RNase A was indistinguishable from native RNase A on paper electrophoresis (Fig. 2), which was evidence for similarity of net charge and size. The synthetic and natural molecules also exhibited the same size by gel filtration on Sephadex G-75.

One of the original and principal criteria (11) for the identity of reduced-reoxidized natural RNase A with the untreated native protein was the equivalence of elution volume on a CM-cellulose column in sodium phosphate buffer. This, together with the recovery of full enzymic activity was the basis for the conclusion that the primary structure of RNase A determined its tertiary structure (11).

Our synthetic enzyme agreed perfectly with native RNase A on CM-cellulose and it possessed 78% of the enzymic activity of the pure natural enzyme.

The data on IRC-50 columns support the conclusion that the synthetic enzyme, after reduction and reoxidation, is similar to the natural enzyme that had been reduced and reoxidized under the same conditions. However, for reasons not understood, there was a discrepancy between the position on this column of native RNase A and normal or synthetic-reduced-reoxidized RNase A. Whether this is an artifact or actually represents a real difference in conformation is not known at this time.

**Enzymic Activity and Substrate Specificity of Synthetic Ribonuclease A**

From the activity data on RNA and C> p there is no doubt that the synthetic RNase A catalyzed the chemical reactions that are characteristic for native RNase A. The most highly purified preparation of the synthetic enzyme was 78% as active against an RNA substrate as the pure, untreated natural enzyme, and when C> p was the substrate the specific activity was 65%. This means that it is possible to synthesize in the laboratory an extremely potent and specific organic catalyst starting from that of the natural enzyme, since it is known that in other instances slight changes in the conformation of RNase can alter the relative activity of the enzyme toward different substrates. Another possible cause might have been differences in the sensitivity of the substrates to the presence of inhibitors of the enzyme. Evidence for inhibitors had come from the data showing an increase in total number of units of activity upon purification of the enzyme and, in addition, the finding that the specific activity against RNA and C> p were more nearly equal for the purified synthetic enzyme than for the crude preparation is also compatible with the idea that inhibitors had been present.

In a series of assays, DNase and RNase T 1 activities of the synthetic RNase A were excluded. The results of the DNase assay (Fig. 10) clearly showed that synthetic RNase A at two different concentrations (4-fold and 40-fold amount of DNase used) had no effect on DNA. With the dinucleotide GpCp as substrate, RNase T 1 catalyzed hydrolysis in accordance with the specificity of this enzyme for the 3' ,5'-phosphodiester bond following a guanosine residue (Fig. 11). As expected, natural RNase A was not active against GpCp and also the synthetic RNase A was completely inactive in this assay. The results of the preceding assay could be confirmed with G> p as substrate. RNase T 1 catalyzed the hydrolysis of the 2' ,3'-cyclic phosphate to give the 3'-phosphate, whereas natural and synthetic RNase A showed no reaction with this substrate. With ApAp as substrate all three enzymes, synthetic RNase A, natural RNase A, and RNase T 1 , were without effect.

Although this is by no means a complete study of substrate specificity the data were all consistent with the specificity to be expected for RNase A. They demonstrated the ability of the synthetic enzyme to cleave both large (RNA) and small (C> p) substrates and to catalyze both the transphosphorylation and hydrolysis steps. They showed the expected requirement for p-ribose instead of p-deoxyribose and for a pyrimidine instead of a purine.

**Calculation of Michaelis Constant**—The close agreement of the K m values of natural and synthetic RNase A (1.20 mg per ml and 1.24 mg per ml, respectively), as calculated from a Lineweaver-Burk plot (Fig. 12), provided good evidence that the enzymic activity exhibited by the synthetic product was a true RNase A activity.

**Preparation of Synthetic S-protein and Synthetic Des-(21-35)S-protein and Their Combination with Natural or Synthetic S-peptide to Give Ribonuclease S and Des-(21-35)-ribonuclease S**

Since the discovery of Richards (64) and Richards and Vithayathil (65) that RNase A is cleaved under certain conditions by subtilisin, primarily at the peptide bond between alanine residue 20 and serine residue 21, several laboratories have been involved in chemical, physicochemical, and x-ray crystallographic studies on the complex, ribonuclease S, formed between S-peptide(1-20) and S-protein(21-124). Recently Wycokoff et al. (10) elucidated the x-ray structure of RNase S at a 2-A resolution and constructed a three-dimensional model, which shows clearly the folding and interactions of the two polypeptide chains. But even such a detailed model cannot answer every question about the relationship between structure and function of the enzyme molecule. Many of these problems can best be attacked through the synthetic approach by replacement or omission of amino acid residues or by specific labeling of the molecule. Extensive studies with synthetic analogues and fragments of the S-peptide have already been performed by two groups (68-69). The first work on investigations of this type with synthetic S-protein is described in this paper. We were interested in the question whether an S-protein fragment lacking the first 5 residues (Resi-
and other parts of the complex. The situation for the next 27, and glutamine 28.

dine 48 has been suggested. For asparagine 24 three hydrogen

tyrosine side chain, interaction with the imidazole ring of histi-
sine 25 then titrates normally. In the RNase S model (10)
the main chain of tyrosine 25 can be hydrogen-bonded with
removal of the S-peptide this interaction is disrupted, and tyro-
side chain carboxylate group of aspartic acid 14 (72). Upon

ammo acid residues, asparagine 24 and tyrosine 25, is different.

data of noncovalent interactions between these 3 serine residues
or activity of RNase S. There was no indication from the x-ray
could be omitted without serious consequences to conformation
that the 3 NHz-terminal serine residues, 21 to 23, of the S-protein
their original work was also found here. Thus, the crude syn-
thetic S-protein and des-(21-25)S-protein gave between 15 and
25% as much activity as the natural S-protein. The control
of synthetic RNase A, which had been partially purified in the
same way, also gave 9% activity after reoxidation.

These studies have shown that approximately equivalent
amounts of RNase activity were generated when the denatured,
reduced form of either synthetic S-protein or synthetic des-(21-
25)S-protein was allowed to refold and reoxidize in the presence
of S-peptide. Although the specific activities ranged between 5
and 9%, (Table IV) they are not considered to be significantly
different from one another because of the several experimental
operations involved. The activities are low for two reasons.
First, this series of experiments was done on synthetic proteins
that had not been highly purified. After cleavage they were
simply passed through a Sephadex G-75 column. Second, when
natural S-protein and S-peptide are reduced and reoxidized under
these conditions they give only about 35% recovery of activity.
This value which was reported by Haber and Anfinsen (69) in
their original work was also found here. Thus, the crude syn-
thetico S-protein and des-(21-25)S-protein gave between 15 and
25% as much activity as the natural S-protein. The control
of synthetic RNase A, which had been partially purified in the
same way, also gave 9% activity after reoxidation.

From their model of RNase S, Wyckoff et al. (71) predicted
that the 3 NHz-terminal serine residues, 21 to 23, of the S-protein
could be omitted without serious consequences to conformation
or activity of RNase S. There was no indication from the x-ray
data of noncovalent interactions between these 3 serine residues
and other parts of the complex. The situation for the next 2
amino acid residues, asparagine 24 and tyrosine 25, is different.
In RNase A and RNase S, tyrosine 25 is one of the 3 "buried"
tyrosyl residues and may be hydrogen-bonded with the buried
side chain carboxylate group of aspartic acid 14 (72). Upon
removal of the S-peptide this interaction is disrupted, and tyro-
sine 25 then titrates normally. In the RNase S model (10)
the main chain of tyrosine 25 can be hydrogen-bonded with
methionine 29, whereas for the phenolic hydroxyl group of the
tyrosine side chain, interaction with the imidazole ring of histi-
dine 48 has been suggested. For asparagine 24 three hydrogen
bridges have been proposed linking it to tyrosine 97, asparagine
27, and glutamine 28.

In des-(21-25)S-protein those five hydrogen bonds are missing.
The data now described (73) have demonstrated experimentally
that on adding S-peptide to either S-protein or des-(21-25)S-pro-
the protein essentially the same ribonuclease activity is generated. It

CONCLUSIONS

We wish to draw three general conclusions from these experi-
ments.

1. A new and independent kind of evidence is provided for the
view that the primary structure of a protein determines its
tertiary structure. The original hypothesis (11, 13) was based on
the observation that native ribonuclease could be reduced and
unfolded in urea and that upon removal of the urea and reoxida-
tion in air it would spontaneously refold into its original
conformation, would re-form the proper disulfide bonds, and
would regenerate its full enzymic activity. The conclusion
that this response was determined solely by the primary struc-
ture of the protein depended on establishing that the unfolded
chain had a completely random form and had lost all of its
secondary and tertiary structure. Otherwise, if a small region
of the molecule were to have retained some information about
its original conformation it might serve as a nucleus to direct
the remainder of the folding of the protein. Most of the data
(1-3) have supported the random coil view, but some (74) have
indicated a structural rigidity in the reduced protein. Whether
the reduced protein retains any of its original structure in urea
solution remains unclear.
In the case of a synthetic protein there is no possibility that any pre-existing information about the natural conformation could be present. The only information put into the synthetic protein is its amino acid sequence. Therefore, if an active enzyme is produced it must be solely a consequence of its primary structure. This was observed for the synthetic ribonuclease A.

2. The synthesis of des-(21–25)S-protein is a beginning toward structure-function studies on whole proteins by use of the synthetic approach. Through chemical synthesis it could be directly demonstrated that amino acid residues 21 to 25 were not required for S-protein and S-peptide to combine, noncovalently, and to fold into an active enzyme. This kind of conclusion can be made with some confidence because these five residues could not be present in the synthetic analogue in any amount and yet it was as active as the parent compound. Structural-activity data on synthetic analogues that prove to be inactive or of very low activity are much more difficult to interpret.

3. Finally we can conclude that it is possible to begin with free amino acids and to assemble them in the laboratory to give a real protein that possesses true enzymic activity.

Note Added in Proof—An antibody neutralisation test has provided further evidence for the similarity between the synthetic and natural RNase A. Samples of synthetic and natural RNase A were incubated with an antibody prepared from rabbits immunized against crystalline bovine pancreatic RNase A and the extent of inactivation was determined by comparing the quantity of acid-soluble nucleotides (20, 21) produced with that in the controls without antibody. The incubation mixture contained: sodium acetate buffer (0.05 M, pH 5.0), 0.34 ml; RNA (1 mg per ml), 1.00 ml; antiserum, 0.12 ml; RNase (0.1 mg per ml), 0.01 ml. The presence of the antiserum caused a drop of absorbance at 260 nm (corrected for the blank) from 0.350 to 0.290 for the natural RNase A and from 0.277 to 0.217 for the synthetic RNase A. Since the assay is linear in this range the data indicate that under the conditions of this single experiment the antibody neutralized essentially equal quantities of the synthetic and natural enzymes.

We are most grateful to Dr. Cecil Yip, Banting and Best Department of Medical Research, University of Toronto, for suggesting this experiment and for supplying the antiserum and the assay.

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